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Changes in the intraisolate genetic structure of *Beet necrotic yellow vein virus* populations associated with plant resistance breakdown

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ABSTRACT

The causal agent of rhizomania disease, *Beet necrotic yellow vein virus* (BNYVV), typically produces asymptomatic root-limited infections in sugar beets (*Beta vulgaris*) carrying the *Rz1*-allele. Unfortunately, this dominant resistance has been recently overcome. Multiple cDNA clones of the viral pathogenic determinant p25, derived from populations infecting susceptible or resistant plants, were sequenced to identify host effects on the viral population structure. Populations isolated from compatible plant–virus interactions (susceptible plant–wild type virus and resistant plant–resistant breaking variants) were large and relatively homogeneous, whereas those from the incompatible interaction (resistant plant–avirulent type virus) were small and highly heterogeneous. All populations from susceptible plants had the same dominant haplotype, whereas those from resistant cultivars had a different haplotype surrounded by a spectrum of mutants. Selection and diversification analyses suggest an evolutionary trajectory of BNYVV with positive selection for changes required to overcome resistance, followed by elimination of hitchhiking mutations through purifying selection.

Introduction

The capability of a plant virus to overcome strain-specific host resistance depends on the frequency of its encounters with the resistant host and its ability to generate and maintain genetic variation. The benyvirus *Beet necrotic yellow vein virus* (BNYVV) is transmitted by zoospores of the plasmodiophorid *Polymyxa betae*. Initially, primary zoospores are released from soil borne resting spores called sporosori. Then, after each rain or irrigation event during the growing season, secondary zoospores are repeatedly released from intracellular zoosporangia (Rochon et al., 2004; Rush, 2003). This cycling production of viruliferous zoospores magnifies the virus encounters with a potential host. BNYVV, however, has a narrow host range and in nature probably infects only a few species of Chenopodiaceae (Hugo et al., 1996; Tamada, 2002). Also, it has limited dispersion inside the plant and root system (Kaufmann et al., 1992; Scholten et al., 1994). Despite that, in susceptible sugar beet cultivars, BNYVV causes a severe disease, named rhizomania, which is characterized by tap root constriction, profuse lateral root proliferation, and leaf chlorosis.

The genome of BNYVV consists of 4 to 5 single-stranded, plus-sense RNA species. RNA 1 and 2 encode the essential elements for replication, encapsidation, and cellular translocation, whereas RNA 3, 4, and 5 are translated into proteins involved in pathogenesis, vector transmission, and suppression of gene silencing (Link et al., 2005; Rahim et al., 2007; Richards and Tamada, 1992; Tamada, 2002). Despite its multi-particulate genome, and the potential of mixed infections with different strains (Koenig et al., 1995), high genetic stability seems to be the norm between spatiotemporally separated populations (Koenig and Lennefors, 2000), and reassortants or recombinants are not frequently found (Koenig et al., 1991; Schirmer et al., 2005). These observations suggest the existence of strong selective constraints on virus diversification, and effective isolation mechanisms operating among sympatric populations. Schirmer et al. (2005) analyzed the genetic variability of 136 viral isolates collected worldwide and found that the RNA 2 encoding CP cistron is highly conserved, whereas p25 (RNA 3) and p26 (RNA 5) genes are more variable with strong positive selection acting on some of their amino acids. The p25 and p26 genes operate synergistically to exacerbate symptoms in certain sugar beet cultivars; they localize in the nucleus and activate transcription (Klein et al., 2007; Link et al., 2005). However, p26 is dispensable in pathogenesis since only some populations from France, England, Japan, Kazakhstan, and China carry its encoding RNA (Harju

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et al., 2005). On the other hand, p25 accounts for most of the rhizomania phenotype expression (Koenig et al., 1991; Tamada et al., 1999), and its amino acids at positions 67–68 and 135 are associated with the capability of some BNYVV populations to overcome *Rz1*-mediated resistance (Acosta-Leal and Rush, 2007).

In the gene-for-gene model of plant–microbe interactions, disease is the result of a compatible interaction (reviewed by de Wit, 1992). Upon the introduction of BNYVV in North America (Al Musa and Mink, 1981), the wild type (WT) virus established a compatible interaction with sugar beet genotypes cultivated at that time. Rhizomania was first identified in California in the mid 1980s and quickly spread to all major production regions in the state. The industry was saved by the introduction of *Rz1* resistant cultivars, but in 2002, rhizomania reappeared in fields of the Imperial Valley of California planted with sugar beets carrying the *Rz1* allele (reviewed by Rush et al., 2006).

In a compatible *rz1*/WT interaction, the virus moves from the initially infected epidermal cells of lateral rootlets to the cortex parenchyma, endodermis, and then xylem parenchyma cells, where the virus forms large clusters (Scholten et al., 1994). From xylem parenchyma, virions might be transported to the tap root through the xylem after the infected cells undergo programmed cell death to form mature tracheary elements (Opalka et al., 1998); a process that may be virus accelerated (Burketová et al., 2003). On the other hand, in the incompatible interaction between *Rz1*-plants and avirulent virus (*Rz1*/AV), the still unknown resistance mechanism(s) is characterized by a reduced amount of cellular virus accumulation rather than a blockage of virus intercellular translocation (Giunchedi and Pollini, 1988; Kaufmann et al., 1992). In field trials, virus content is directly proportional to symptom severity and the number of recessive *rz1* alleles carried by heterozygous resistant genotypes (Wisler et al., 1999). Also, the reduction of virus accumulation is more pronounced in tap than lateral roots (Tamada et al., 1999). In the Imperial Valley of California, the breakdown of resistance by emerging resistance-breaking (RB) variants is characterized by severe disease expression associated with high virus titer (Liu et al., 2005) and shifts in the viral p25 motifs from $A_{67}C_{68}D_{135}$ or $A_{67}L_{68}D_{135}$ to $V_{67}L_{68}E_{135}$ or

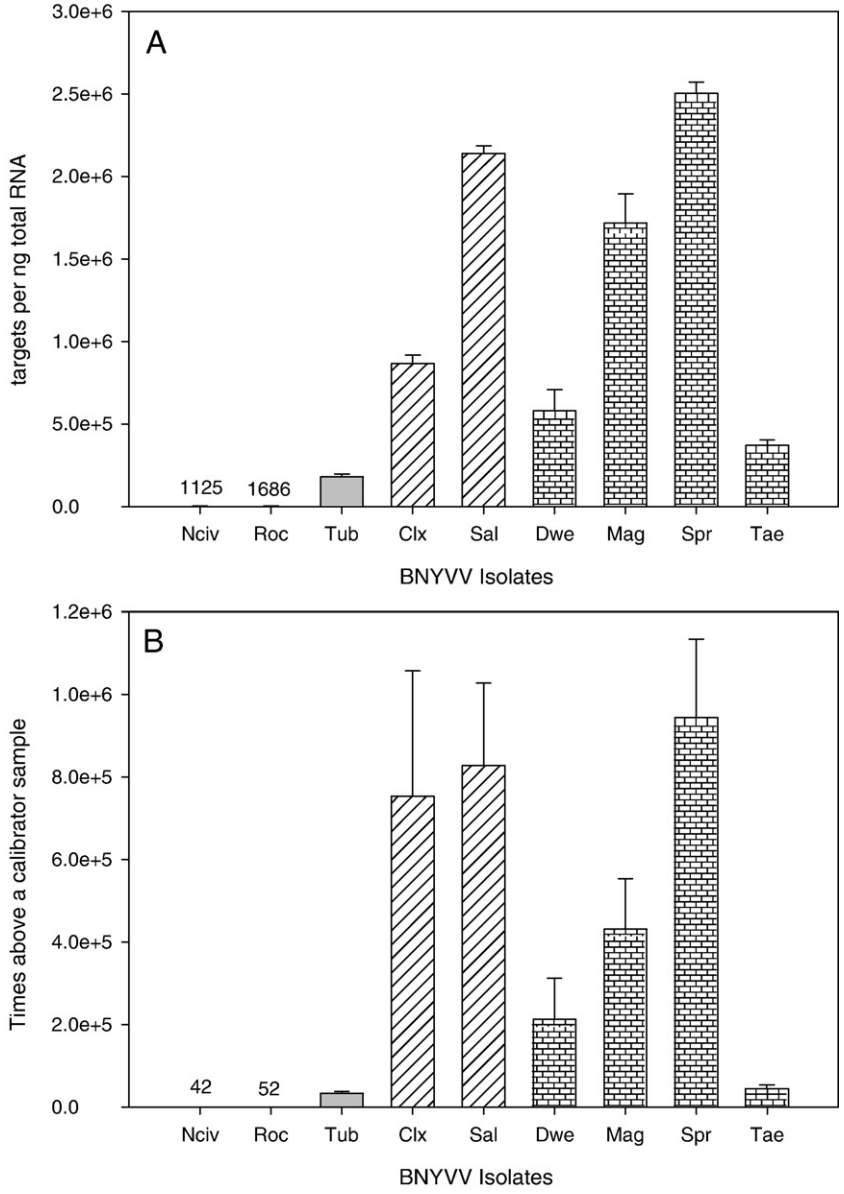

Fig. 1. Realtime RT-PCR absolute (A) and relative (B) virus quantification of isolates involved in the sugar beet–BNYVV interactions: resistant–avirulent (*Rz1*/AV, solid bars), susceptible–wild type (*rz1*/WT, striped bars), and resistant–resistant breaking (*Rz1*/RB, brick bars). Mean values with standard deviation of three repetitions per isolate.

$V_{67}C_{68}E_{135}$ (Acosta-Leal and Rush, 2007). At present, both incompatible *Rz1*/AV and compatible *Rz1*/RB interactions co-exist in many fields of the Imperial Valley and possibly in other parts of the world.

A viral population whose specific genetic structure (diversity and frequency of haplotypes), rather than its dominant haplotype, is selected to predominate in a definite environment is referred to as a quasispecies (Eigen, 1996; Holmes and Moya, 2002). Only a few viruses, such as *Vesicular stomatitis virus* (de la Torre and Holland, 1990; Novella et al., 1995), phage φ6 (Burch and Chao, 2000), or *Human poliovirus* (Vignuzzi et al., 2006), have been analyzed thoroughly enough to determine if they meet the requirements of the quasispecies concept. Consequently, most references to viral quasispecies are based solely on a description of their genetic heterogeneity. Similarly, there are few examples of plant viruses or viroids that have been characterized at the intrapopulational level. For those that have, however, the data should be interpreted with caution, because in most instances the agent was mechanically transmitted before analyses, which might have disturbed the natural population structure. Kurath et al. (1992) provided the first evidence that a viral RNA population infecting a single plant can be highly heterogeneous (11.3×10^{-4} nucleotide substitutions per site) and composed of infectious and non-infectious viral genomes. Since then, high intrinsic heterogeneity also has been found in tobamoviruses (Kim et al., 2005), potyviruses (French and Stenger, 2005; Hall et al., 2001), geminiviruses (Isnard et al., 1998), *Cucumber mosaic virus* (CMV, Schneider and Roossinck, 2001), *Citrus tristeza virus* (Ayllón et al., 1999; Kong et al., 2000), *Citrus leaf blotch virus* (CLBV, Vives et al., 2002) and remarkably in viroids (Ambrós et al., 1998 and 1999). In general, most infecting viral populations might be composed of an arrangement of genomes distinguished from each other by at least one mutation. Nonetheless, when consensus sequences from different isolates are compared, the majority of them are almost identical (Choi et al., 2001; Fraile et al., 1997; Marco and Aranda, 2005). This situation has been explained by the hypothesis that purifying selection is the predominant evolutionary force imposed on plant virus populations that have reached a state of equilibrium (reviewed by García-Arenal et al., 2001).

Theoretically, the genetic structure of viral populations influences their biological properties, such as host range, adaptation, pathogenicity, fitness, transmissibility, evolutionary potential, etc., but few efforts have been made in plant virology to test this hypothesis. For instance, it has been found that the spectrum of mutants can be altered by the host environment (Ayllón et al., 1999; Hall et al., 2001; Schneider and Roossinck, 2001), but this change has not been systematically correlated against any other characteristic of the host or the viral infecting population. Instead, most studies have focused on identifying the mutations that became fixed in the consensus sequence and confer novel properties to the virus, such as those that allow them to overcome resistance (reviewed by Harrison, 2002). Our working premise has been that, although these mutations are the final determinants, their emergence is conditioned by the behavior of the parental quasispecies. To our knowledge, this is the first study where the populational genetic structure of a soil borne plant virus is determined. Moreover, it is the first investigation where the natural genetic composition of viral populations is associated with a biological property of the virus; in this case, their genetic compatibility with the host.

Results

Disease expression, virus titer, and viral p25 motif are associated

With the exception of BNYVV-Chr, all isolates included in this study were collected from 3–5 plants naturally infected in the field and clustered within a localized sampling area. These composite samples were grouped during the analyses according to plant response and host genotype. Thus, the compatible *Rz1*/RB group was composed of resistant breaking isolates: Dwe, Mag, Spr, and Tae, which were taken from *Rz1* resistant cultivars showing severe rhizomania. The incompatible *Rz1*/AV group consisted of avirulent Nciv, Roc, and Tub isolates that were collected from asymptomatic *Rz1*-resistant cultivars. Finally, the compatible *rz1*/WT group was comprised of wild type Clx and Sal isolates obtained from diseased susceptible cultivars, and the Chr isolate that was baited out from rhizosphere soil collected in Texas from a symptomatic susceptible cultivar in 1991 (more specifics of the isolates can be found in Materials and methods). Thus, each isolate was considered a representative sample of a viral population. Consequently, the terms "isolate" and "population" could be considered to be interchangeable. However, to avoid confusion, we reserved the term "isolate" to refer only to the isolated sub-population represented by the cloned molecules of the sample.

The use of realtime absolute and relative RT-PCR quantification revealed considerable variation in virus content among populations (Fig. 1). Despite that, in compatible interactions the amounts of amplifiable particles were 10^2 to 10^4 times higher than in the incompatible *Rz1*/AV interaction. An exception was the virus titer compared between avirulent Tub and resistance-breaking Tae populations, where a significant difference of around 2-fold was detected only by absolute quantification. In general, disease expression was associated with a virus content of at least 3×10^5 targets per nanogram of total RNA extracted from mature plants grown in the field. In some plant roots, the amount of virus was as high as 2.5×10^6 targets ng^{-1} of total RNA.

As expected from previous results (Rush et al., 2006), only populations from the *Rz1*/RB interaction were comprised of dominant haplotypes encoding the resistant breaking $V_{67}L_{68}E_{135}$ or $V_{67}C_{68}E_{135}$ p25 motifs. Some of these RB populations also contained haplotypes

Table 1
Genetic diversity of virus isolates collected from compatible (*rz1*/WT, *Rz1*/RB) and incompatible (*Rz1*/AV) sugar beet–BNYVV interactions

Interaction[a]	Isolate	Sequenced clones	Mutation frequency ($\times10^{-4}$)	Nucleotide diversity[b] ($\times10^{-3}\pm$S.E.)	p25-motif[c]	
					Dominant	Co-existing
rz1/WT	Chr	13	1.6 (2/12,272)	0.33±0.22	ACD	–
	Clx	10	0 (0/9,220)	0	ACD	–
	Sal	10	5.4 (5/9,220)	1.06±0.42	ACD	–
Rz1/RB	Dwe	14	8.9 (17/19,138)	1.52±0.4	VLE	ALD
	Mag	17	6.8 (11/16,048)	1.58±0.5	VLE	–
	Spr	9	2.3 (2/8,541)	0.47±0.3	VLE	–
	Tae	23	5.4 (17/31,441)	1.94±0.6	VCE	ALD,VRE
Rz1/AV	Nciv	10	11.5 (11/9,610)	3.21±1.0	ALD	ACD
	Roc	17	10.3 (24/23,239)	2.48±0.5	ALD	–
	Tub	10	16.8 (23/13,670)	4.40±1.2	ALD	–

[a] The compatible plant–virus interactions between the susceptible (*rz1*) or resistant (*Rz1*), and the wild type (WT) or resistance-breaking (RB) isolates were characterized by severe rhizomania disease. In contrast, the incompatible *Rz1*/AV interaction was asymptomatic.
[b] Estimated by the Kimura 2-parameter model as implemented in MEGA 3.1 software (Kumar et al., 2004), and standard error (S.E.) estimated by 500 replicates bootstrapping.
[c] Amino acids predicted at positions 67, 68 and 135 of the p25 cistron of the viral RNA 3.

that encoded the avirulent $A_{67}L_{68}D_{135}$ motif and an undetermined $V_{67}R_{68}E_{135}$ motif (Table 1). Also the avirulent Nciv population contained few particles encoding the wild type $A_{67}C_{68}D_{135}$ motif. Given the composite nature of the root-tissue samples, it is unknown if these minor haplotypes were co-infecting the same plant tissue, but their spatiotemporal coexistence suggested an evolutionary lineage from WT to AV to RB populations.

The populations are phylogenetically structured by their host compatibility

A viral segment of RNA 3, including the p25 cistron, was amplified by high-fidelity RT-PCR and cloned. Sixty-one different haplotypes were identified out of 133 nucleic acid sequences analyzed (9 to 23 clones per isolate). Phylogenetic analyses revealed that these haplotypes were clearly structured based on their plant–virus interaction group (Fig. 2). However, there were some cases where a haplotype of one population was more closely related to haplotypes of another population instead of its own. This haplotype overlapping was common in the *rz1*/WT group despite the fact that these populations were the most spatiotemporally separated [Minnesota (Clx) and California (Sal), 2005; and Texas (Chr), 1991]. This finding supports the notion that they belong to a single North American BNYVV macropopulation. Also, sequences from this group were the most similar to some of the GenBank sequences used as an outgroup. The lineage that comprised populations of the incompatible *Rz1*/AV group contained two haplotypes from the *Rz1*/RB group. The genetic similarity between these overlapping haplotypes supported the notion that California RB variants evolved from avirulent progenitors from the same region rather than from an ancestor that was externally introduced.

Analysis of molecular variance (AMOVA) indicated that each interaction group (Table 1) represents a separate population of sequences ($p<0.001$), a result congruent with that of the phylogenetic analysis. However, some isolates within the same interacting group were found to belong to the same population. For instance, within the *rz1*/WT group, none of the isolates were significantly different from

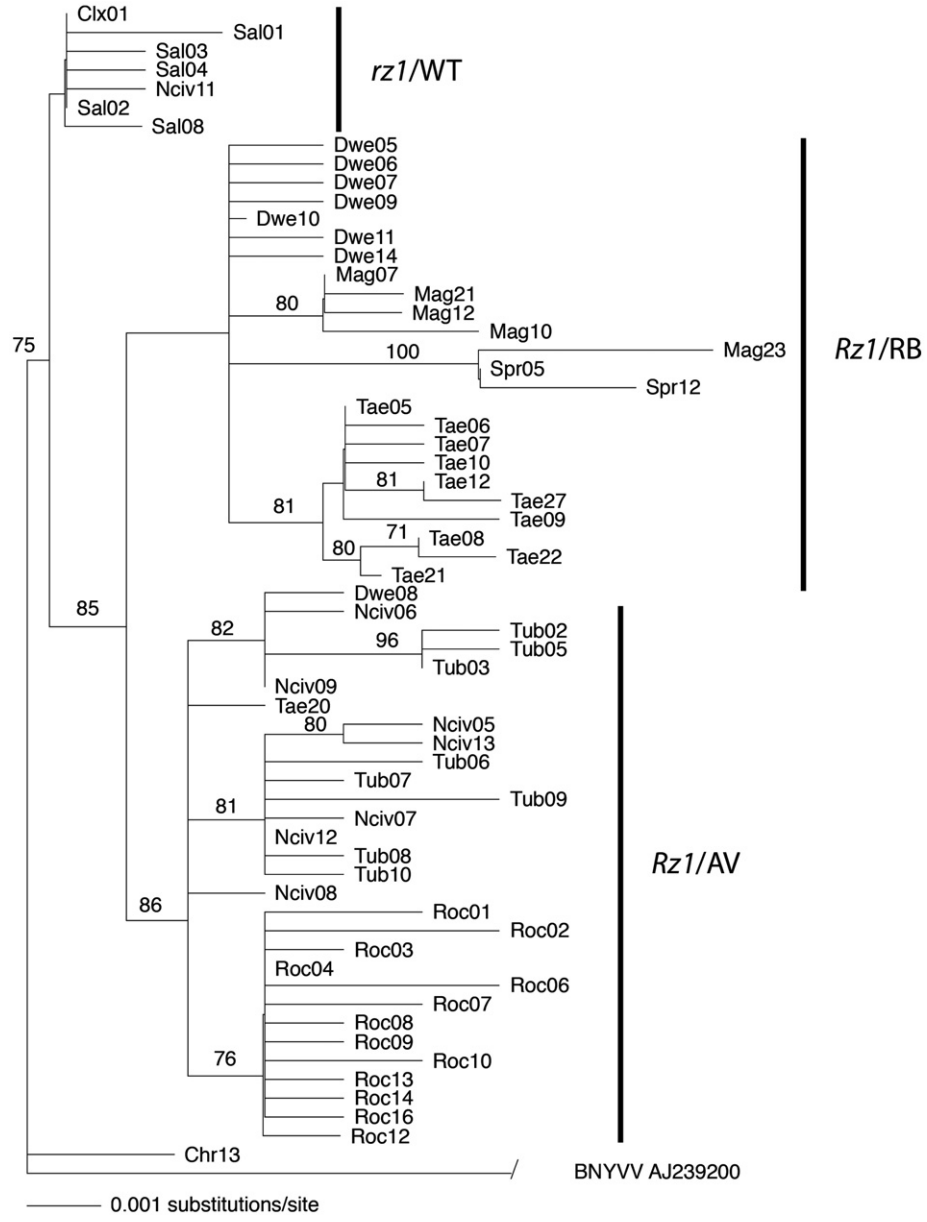

Fig. 2. Phylogenetic relation of 61 haplotypes of BNYVV RNA 3 p25 region found in populations differentiated by their interaction with susceptible (*rz1*) and resistant (*Rz1*) cultivars of sugar beets.

each other. Similarly, within the *Rz1*/AV group, no significant difference existed between the NCiv and Tub isolates, but the Roc isolate represented a distinct population ($p<0.001$). In contrast, all *Rz1*/RB isolates represented distinct populations ($p<0.001$). Thus, the populations from the *Rz1*/RB interaction group may not be derived from a recent common ancestral strain or they have evolved separately to such an extent that the phylogenetic signal of common ancestry was obscured.

Plant–virus compatibility affects genetic diversity within and between populations

When the overall nucleotide diversities of the sequences included in each plant–virus interaction group were compared, only the less diverse *rz1*/WT group was significantly different from the others (Table 2). However, when the overall diversity was broken down by diversity within and among populations, the intrapopulational diversity was highest (3.07×10^{3}) in the incompatible *Rz1*/AV interactions, whereas the differences among populations were greatest (2.43×10^{3}) in the *Rz1*/RB group. Individually, the most heterogeneous population was the avirulent BNYVV-Tub and the most homogeneous was the wild type BNYVV-Clx (Table 1). A slight inverse correlation was detected between virus titer and nucleotide diversity when BNYVV-Tub was treated as an outlier ($R^2=0.67$). Otherwise, these two variables were not correlated ($R^2=0.36$).

The landscape of haplotypes: more sequence space is explored in resistant plants

The genetic variability found in each plant–virus interaction group was further characterized by the type of mutations. Thus, fixed mutations corresponded to polymorphic sites detected at the level of consensus sequences where the specific nucleotide substitution was present in all the clones of a given isolate. A singleton was a mutation found solely in one clone of the entire interaction group. Parsimony informative mutations were, consequently, the rest of the nucleotide substitutions detected in the group. All the mutations detected in *rz1*/WT were singletons, as well as 84% of the mutations in the incompatible *Rz1*/AV interaction. In contrast, the proportion of fixed mutations was 10 times higher in *Rz1*/RB than in its avirulent counterpart (Table 3). Thus, singletons accounted for most of the intrapopulational diversity detected in AV populations, whereas fixed mutations explained most of the variation found among RB populations.

The 61 haplotypes, originally identified using DnaSP software, were redefined as 19 super-haplotypes by excluding the variation introduced by singletons (Table 4). The rationale was that fixed and parsimony informative mutations might have a bigger evolutionary value than singletons because they, most likely, have been already propagated into the virus progeny (French and Stenger, 2003). Whereas super-haplotype B was the dominant sequence in all the populations composing the *rz1*/WT group, each isolate taken from resistant *Rz1*-plants was configured by a distinct dominant super-haplotype surrounded by lower frequency variants (Fig. 3). The number of super-haplotypes found in each population was only slightly affected by the total number of sequenced clones per population ($R^2=0.37$), and, clearly, the diversity of dominant super-haplotypes was greater in resistant than in susceptible plants.

Table 2
Genetic distances among clones and among isolates of the same plant–virus interaction group

Parameter[a]	Interaction group[b]		
	rz1/WT	*Rz1*/RB	*Rz1*/AV
No. of isolates (no. of clones)	3 (33)	4 (63)	3 (37)
Overall nucleotide diversity ± S.E. ($\times10^{-3}$)	0.45 ± 0.1	3.69 ± 0.9	3.57 ± 0.7
Mean diversity within isolates ± S.E. ($\times10^{-3}$)	0.46 ± 0.1	1.26 ± 0.2	3.07 ± 0.5
Mean interisolate diversity ± S.E. ($\times10^{-3}$)	0.01 ± 0.02	2.43 ± 0.8	0.5 ± 0.4

[a] Nucleotide diversities estimated by the Kimura 2-parameter model as implemented in MEGA 3.1 software (Kumar et al., 2004), and standard error (S.E.) estimated by 500 replicates bootstrapping.
[b] Each plant–virus interaction group was independently analyzed and each isolate was treated as an individual population.

Table 3
Types of mutation detected in each plant–virus interaction group based on their frequency inside the group

Types of mutations[a]	Group		
	rz1/WT	*Rz1*/RB	*Rz1*/AV
Proportion of fixed mutations	0	0.22	0.02
Proportion of parsimony informative mutations	0	0.11	0.14
Proportion of singletons	1.0	0.67	0.84
Overall number of mutations	7	45	57

[a] By aligning all the sequences that belong to the same interaction group, singletons and parsimony informative mutations were detected by DnaSP v4.10.7 (Rozas et al., 2003). Then, those parsimony informative mutations that were detected in all the clones of at least one isolate were subtracted and counted as fixed mutations.

Genetic drift, positive, and purifying selection operate during distinct steps of the virus evolutionary trajectory

To determine the selective value of the observed fixed and parsimony informative mutations, the ratio of nonsynonymous substitutions per nonsynonymous sites versus synonymous substitutions per synonymous sites ($d_{NS}/d_S=1$ for neutrality, >1 for positive, and <1 for negative selection) was estimated in pairwise comparisons among consensus sequences with or without the parsimony informative mutations. Neutrality was detected between AV isolates suggesting that they have diversified by genetic drift. Positive selection was evidenced in the mutations that define the evolutionary trajectory of BNYVV from WT to AV to RB populations, whereas all the other mutations in the RB isolates seemed to be under purifying selection. Codons under selection were identified using Internal Fixed Effects Likelihood (IFEL, Kosakovski Pond et al., 2006) analysis. This analysis examined specific lineages within the phylogeny of the sequences and calculated the probability of selection at each codon. IFEL analysis of the complete data set indicated that positive selection was likely on the codons 67, 68, and 135. However, the significance for positive selection at these codons was weak, with $p<0.1$ but >0.05, for all three codons. This result suggests that more haplotypes were needed to provide robust support for this analysis. To evaluate the hypothesis that positive selection is occurring on the avirulent populations, IFEL analysis was also performed on the data set after removal of RB haplotypes. This analysis indicated robust support ($p<0.05$) for selection on codon 68 only. Thus, positive selection at codon 68 may be important for initial adaptation of AV populations to the resistant host environment.

Discussion

BNYVV populations derived from susceptible (*rz1*) sugar beets were composed by the same dominant WT haplotype surrounded by few mutants. However, in resistant *Rz1*-cultivars, the scenario was completely different: each population contained a different dominant haplotype that was enclosed, in most cases, with a broad spectrum of mutants. Moreover, in the incompatible interaction between *Rz1*-plants and avirulent populations (*Rz1*/AV), the genetic diversity was 2–3 times higher than in the compatible interactions *rz1*/WT and *Rz1*/RB populations. Collectively, these data suggest that sugar beet cultivars carrying the *Rz1* allele altered the genetic structure of BNYVV in a way that promoted the generation and selection of RB variants.

Table 4
Super-haplotypes of BNYVV defined by the parsimony informative nucleotide substitutions found in the p25 cistron without taking the singletons in consideration

Haplo-type	p25-motif	No.[a]	Position of the nucleotide in the deducted open reading frame (660 nucleotides long)															
			9	12	62	134	153	189	200	202	203	233	256	285	323	405	553	571
A	ALD	2	U	A	C	A	G	A	C	C	U	U	C	U	A	U	G	U
B	ACD	33								U	G							
C	ACD	1				G				U	G							
D	VLE	15							U							G		
E	ALD	5			U													
F	VLE	13							U				U			G		
G	VLE	1							U				U		G	G		
H	VLE	1	C	U		G		U	U							G		
I	VLE	9	C	U				U	U							G		
J	VCE	11							U	U	G					G		
K	VCE	5							U	U	G	C				G		
L	VCE	1							U	U	G				G	G		
M	VCE	4					C		U	U	G					G		
N	VRE	1							U		G	C				G		
O	ALD	2												C			A	
P	ALD	8															A	
Q	ALD	15																
R	ALD	2																G
S	ALD	4			U													

Note. the super-haplotype A and Q were differentiated by a mutation outside the p25 ORF.
[a] A total of 133 sequenced clones were analyzed with the software DnaSP (Rozas et al., 2003) to identify a total of 19 super-haplotypes using only the parsimony informative sites (no singletons considered).

Relationship between plant resistance breakdown and the viral p25 motif

The breakdown of plant resistance to viral diseases does not always occur by changing the genetic makeup of the pathogen. Plant resistance can also be overcome in a non-hereditable way and under specific environmental conditions, such as increased inoculum concentration (Tu, 1989), mixed virus infections (García-Cano et al., 2006; Scheets, 1998), or physiological plant stress (Chandra-Shekara et al., 2006; Holmes, 1937). The first indications of breakdown of the sugar beet *Rz1*-gene were the reemergence of rhizomania in the Imperial Valley of California during 2002, and the detection of BNYVV in high concentration in plants grown in soil from that region (Liu et al., 2005). Later, a shift in the viral genotype was associated with disease expression in the field (Acosta-Leal and Rush, 2007), which supports the idea that RB variants have evolved in the Imperial Valley, rather than a breakdown in resistance caused by environmental factors. In this study, we found stronger support for resistance breakdown through the emergence of RB variants, as indicated by correlations among disease expression in the field, virus content in *Rz1*-plants, and viral p25 motif (Fig. 1 and Table 1). Moreover, selection analyses indicate that changes at amino acid positions 67–68 and 135 have been positively selected in resistant plants. Despite that, conclusive evidence will be obtained only by reverse genetics experiments. Nevertheless, considering that p25 may operate by interacting with other viral proteins (Klein et al., 2007); its expression in a heterologous viral background (i.e., by using infectious transcripts of RNA 1 and 2 from another strain) might not produce the predicted results. Instead, the high similarity between some AV and RB variants detected in this work, such as Nciv versus Dwe, makes them good candidates for this type of experiment.

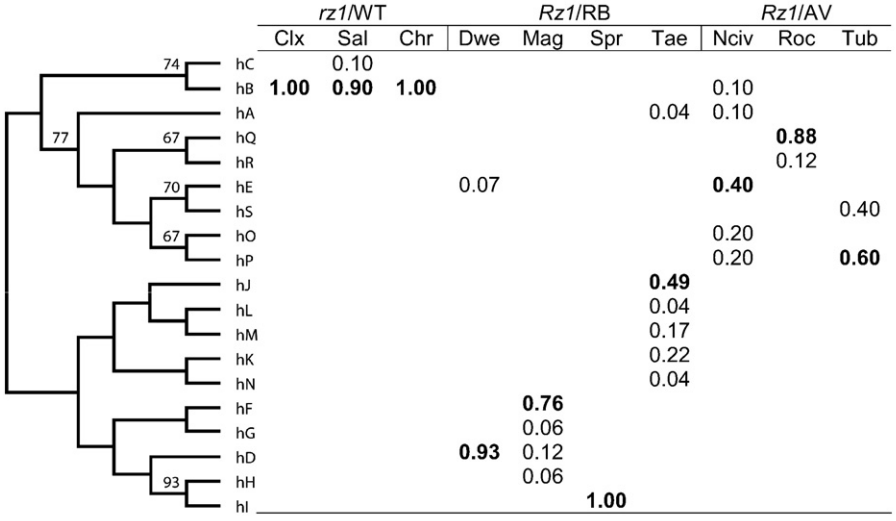

Fig. 3. Phylogenetic landscape of the relative frequency of super-haplotypes of BNYVV RNA 3 p25 cistron grouped according with their plant–virus interaction. The proportion of each super-haplotype was calculated in relation with the total number of sequenced clones of its corresponding population. The proportion of the dominant super-haplotypes is in bold.

Relationship between plant resistance breakdown and the virus population structure

The differential genetic diversity found between RB and AV populations was only correlated with disease expression and, consequently, virus content. The possibility that this association was coincidental is remote since the composite samples were collected in different years and from different fields that were several miles apart in the Imperial Valley. Despite that, all AV populations exhibited higher mutation frequencies and nucleotide diversities than all the RB populations. Therefore, the cause of this genetic diversity–disease correlation is most likely attributable to factors that determine the plant–virus compatibility rather than environmental conditions.

Plant viruses produce more genetic variability in some host species than in others. For instance, Rodríguez-Alvarado et al. (1995) found that CMV infecting a field crop of pepper (*Capsicum annuum*) contained an unusually high diversity of haplotypes. Later, in a comparative study, Schneider and Roossinck (2001) noted that CMV was more heterogeneous in pepper than in *Nicotiana benthamiana*. Recently, Pita et al. (2007) provided evidence that the replicative fidelity of CMV is lower in pepper than in tobacco (*Nicotiana tabacum*). Based on these findings, there is a possibility that the replicative error rate of AV populations was higher in resistant than in susceptible sugar beets.

Another mechanism that could increase the genetic diversity of an infecting virus population is an incompatible host–virus interaction that selectively restricts accumulation of the dominant haplotypes. Under this hypothetical situation, the chances of selecting low-frequency haplotypes during cloning would be greater than drawing them from large populations dominated by few high-frequency haplotypes. *Rz1* resistance reduced the size of infecting AV populations, and may preferentially have restricted accumulation of dominant haplotypes. If this was the cause of the differential nucleotide diversity observed in this study, an inversely proportional relationship between diversity and virus titer would have been expected. However, this putative relationship was not clearly evidenced, and it will require analyzing larger numbers of populations, and sequences per isolate, to reach a conclusion on this issue.

The highest variability within AV populations was not translated into the highest diversity among them. Instead, the highest diversification among populations was observed in the *Rz1*/RB group. Similar results were obtained by Vives et al. (2002) who found that the interisolate variation of CLBV was higher than the variation within isolates. This peculiar pattern has been observed, for instance, during the introduction of a virus into a new ecosystem and is attributed to a founder effect where each new infection passes through a bottleneck event (reviewed by García-Arenal et al., 2001). Therefore, the higher interpopulational variation detected in BNYVV RB populations suggests that they, like any other plant virus, went through bottleneck transmission events during their spread in the field. This result is somewhat surprising because roots of sugar beets are normally infected by a large number of viruliferous zoospores and consequently, a bottleneck effect produced by a single transmission was expected to be minimized by multiple infections of the same plant root.

A putative viral evolutionary trajectory to overcome plant resistance

Given the replicative strategy of viruses (i.e. high mutation rate during the synthesis of numerous relatively small genomic molecules), genetic variation is generated beginning with the first replication cycle of a viral clone (Cuevas et al., 2005), and consecutive infection cycles might create unique viral lineages following separate evolutionary trajectories. During this process, the genetic structure of spreading populations is shaped by numerous factors, such as environmentally imposed selective constraints, infection opportunities for selectively similar virus genotypes, and molecular interactions between co-infecting haplotypes. The evolutionary path followed by BNYVV to overcome *Rz1*-mediated resistance in the Imperial Valley seems to be as follows: during the incompatible *Rz1*/AV interaction, the virus accumulates large genetic variation, most of it selectively neutral and in the form of singletons. It potentiates the virus to diversify by random drifting during its replication and spread in asymptomatic plants. However, this diversification of AV populations is limited by positive selection at the amino acid position 68. Once additional emerging mutations provide adaptive advantages, as those characterizing the compatible *Rz1*/RB interaction, these changes are positively selected and become fixed in the virus population. Finally, purifying selection or selective sweeps eliminate most of the accumulated variation that was carried on as hitchhiking mutations.

The long evolutionary stasis of BNYVV, as suggested by the structure of the *rz1*/WT macro-population, followed by a short period of extensive genetic diversification (i.e., the period during virus adaptation to infect *Rz1*-plants) agrees with the punctuated equilibrium model of speciation proposed by Eldredge and Gould in 1972 (reviewed by Seaborg, 1999). This evolutionary model explains the pandemic diversification of *Vesicular stomatitis virus* on the American Continent (Nichol et al., 1993), and predicts the emergence of regionally adapted variants, some of them more pathogenic than others. Therefore, a control strategy that might delay this process would be the deployment in the field of a collection of resistant varieties with distinct defense mechanisms, including transgenically modified plants, which might cause extreme diversification of BNYVV and, consequently, a loss of fitness.

Materials and methods

Field isolates

BNYVV rarely infects foliar tissue, but root-infected plants normally develop generalized chlorosis during maturation facilitating the identification of plants with rhizomania (Rush et al., 2006). In this way, apparently healthy and diseased sugar beets (*Beta vulgaris* ssp. *vulgaris*) were identified and then asymptomatic or symptomatic lateral roots were collected from 3–5 plants in the same condition. Most of the isolates included in this study were from the Imperial Valley of California. These include samples collected in 2005 from four fields that were several miles apart from each other and resulted in isolates Dwe, Mag, Spr, Tae, and Nciv (same field as Mag), and samples collected in 2006 from two distantly separated fields (isolates Roc and Tub). Isolates from outside the Imperial Valley came from Salinas Valley, CA in 2005 (Sal was kindly provided by Dr. Robert Lewellen, USDA-ARS), Climax, MN collected the same year (Clx), and isolate Chr that was baited from a soil sample collected in Texas during 1991. All the samples from the Imperial Valley were from non-isogenic *Rz1*-cultivars 'Phoenix' or 'Beta 4430,' whereas the rest of the samples were from susceptible 'Roberta' or 'Cristal 765' varieties.

Total RNA extractions from root tissue

RNA was extracted from root tissue using the RNAqueous®-Mini kit (Ambion Inc. Austin, TX) with the following modifications: plant tissue (0.1 to 0.2 g) was frozen with liquid N_2, powdered inside a 2.0 ml microfuge tube, and resuspended in 1.5 ml of the Ambion Lysis/Binding Solution. The supernatant obtained after centrifugation (16,000 ×g for 10 min) was mixed with 1.5 ml of 64% ethanol and passed through the glass fiber filter (Ambion Spin cartridges cat# 10051g) using a vacuum manifold. The filter was dried by centrifugation (16,000 ×g for 1 min) and washed with 1.5 ml of Ambion Solution #1 using the vacuum manifold and then, with 2.0 ml of Ambion Solution #2/3 in the same manner. At the end, the filter was dried again by brief centrifugation and transferred to a 1.7 ml microfuge tube where the RNA was eluted in 50 µl of

preheated (80 °C) Ambion Elution Solution during a centrifugation at 16,000 ×*g* for 30 s.

RT-PCR, cloning, and sequencing

First strand cDNA was synthesized using the Omniscript® reverse transcriptase kit (Qiagen Inc., Valencia, CA) according to manufacturer recommendations. Total RNA (0.5 µg) was denatured at 65 °C for 5 min before annealing with 0.5 µg of oligo-dT_{12-18} primer and incubating at 37 °C 1 h^{-1} for cDNA polymerization. PCR was performed separately using Platinum® *Taq* high-fidelity polymerase (Invitrogen, Inc., Carlsbad, CA) and 5.0 µl of the reverse transcription products. Forward, F29 (5′-TTAATCCAAGTACCTCGTCT-3′) and reverse, R30 (5′-TTGAAATTGTGATAACTCTAA-3′) or R32 (5′-CATGTAACCGGCTGTG-GAACC-3′) primers were added at 0.5 µM each, and the rest of the reagents were incorporated at concentrations recommended by the manufacturer. The PCR was run for 25 cycles of denaturing at 94 °C 30 s^{-1}, annealing at 55 °C 30 s^{-1}, and extending at 68 °C 1 min^{-1} for F29–R30 primers, or annealing at 58 °C 30 s^{-1}, and extending at 68 °C 1:30 min^{-1} for F29–R32 primers. The amplified DNA fragment, composed of 974 or 1367 base pairs, was cleaned using the QIAquick kit (Qiagen Inc., Valencia, CA), quantified by spectrophotometry, and recombined with pCR®-Blunt (Invitrogen, Inc., Carlsbad, CA) vector. Plasmid DNA was extracted from individual clones using the QIAprep Spin Miniprep Kit (Qiagen, Inc.) and sequenced in both directions by a commercial company to analyze the genetic composition of the infecting populations. To minimize experimental error, cloned DNA was never exposed to mutagens (i.e., UV or ethidium bromide) and the bacterial colonies were not re-plated.

Quantitative realtime RT-PCR

BNYVV titers in infected tissue were estimated by both absolute and relative realtime RT-PCR quantifications. In both systems, the primers 50F (5′-CCGTTTTCCACAGACACTAACTATGTA-3′) and 51R (5′-TGCT-AACCCTGAATCAGTTAAAGTACTT-3′) plus TaqMan probe NYCP (6FAM-TGCACTTGTGTTATATGTTAATCTGTCTGACCCAG-TAMRA) were incorporated in one-step RT-PCR to target the core of the CP cistron in RNA 2. The realtime reactions were performed by an ABI Prism 7000 system (Applied Biosystems, Inc., Foster City, CA) using the following parameters: reverse transcription at 48 °C 30 min^{-1}, followed by reverse transcriptase inactivation at 95 °C 10 min^{-1} and then, amplification during 40 cycles of denaturing at 95 °C 15 s^{-1} and annealing at 60 °C 1 min^{-1}.

For absolute viral RNA quantification (no. of targets ng-1 of total RNA), serial dilutions of pure cRNA encoding the target BNYVV CP region were used as standards. This 814 bp transcript was prepared by *in vitro* transcription (Megascript T7 kit, Ambion, Inc.) of plasmid DNA. The concentration (pg μL^{-1}) of the initial suspension was estimated by spectrophotometry then, the mass converted to number of targets by the formula $(pg \times 6.023 \times 10^6)/2.768$. Relative quantities were estimated by the ΔΔCt method (Livak and Schmittgen, 2001) using 18S ribosomal RNA as an endogenous reference (Applied Biosystems Inc.), and a plant RNA sample with the lowest detectable virus titer as calibrator. In both systems, the experimental unit was 20 ng of totally extracted RNA (isolate) per 25 µL reaction. Each RNA sample was loaded in three plate wells (repetitions), and all the isolates were tested during two independent assays with similar results.

Nucleic acids sequence analyses

The basic processing of sequences, such as assembling, correction, and alignment, were performed with the DNAStar package v4.0 (Dnastar Inc., Madison, WI), and the chromatograms were inspected with Sequence Scanner v1.0 (Applied Biosystems, Inc.) to verify the presence of mutations. Out of 133 sequenced clones, 61 different haplotypes were found in this study and their sequences are been deposited in public databases. These haplotypes were phylogenetically analyzed using the Neighbor-Joining approach as implemented in PAUP* 4.0b10 (Swofford, 2002). The BNYVV p25 sequence AJ239200 was included as an outgroup to root the analysis. The alignment consisted of 944 characters, of which 80 were variable. A jackknife procedure, with 20% deletion for each replication and 1000 replicates, was used to evaluate the resulting tree. Population differentiation among interaction groups and isolates was determined by AMOVA using Arlequin ver. 3.1 (Excoffier et al., 1992, 2005) with 16,000 permutations. Population comparisons were made using Reynolds' distance (Reynolds et al., 1983).

The genetic structure of each isolate (population) was analyzed in relation to its nucleotide diversity and genetic distances among clones and among isolates using the Kimura 2-parameter model as implemented in MEGA 3.1 software (Kumar et al., 2004). The standard errors were estimated by 500 replicates of bootstrapping. Some measurements were corroborated with DnaSP v4.10.7 (Rozas et al., 2003), which also was used for identification of singletons, parsimony informative sites, and haplotypes. The definition of super-haplotypes (Table 4) was done by DnaSP by entering a file with the total number of sequences (133), and then, manually editing them to include only the mutations found in the originally detected 19 parsimony informative sites (sixteen of them inside the p25 open reading frame). Tests of selection operating on the p25 gene (219 coding codons) were performed by estimating the ratio of non-synonymous substitutions per nonsynonymous sites versus synonymous substitutions per synonymous sites (d_{NS}/d_S). Selection analysis was conducted between consensus pairs of sequences, with and without the parsimony informative mutations, following the Nei and Gojobori's model as implemented in DnaSP. Then the level of statistical significance to reject neutrality ($d_{NS}=d_S$), versus positive ($d_{NS}>d_S$) or purifying ($d_{NS}<d_S$) selection, was determined by the *Z*-test according with the same model but executed in MEGA 3.1. The variance was calculated by bootstrapping at 500 repetitions. Selection was also analyzed at the individual codons level by using the internal fixed effects likelihood (IFEL) method (Kosakovski Pond et al., 2006) as implemented in the HyPhy software package (Kosakovski Pond et al., 2005) on the Datamonkey server (http://www.datamonkey.org).

Nucleotide sequence accession numbers

The nucleotide sequences of the 61 haplotypes of the BNYVV p25 region described in this study are available in GenBank and mirror databases (accession numbers EU480492 to EU480552).

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